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CS Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA.
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AU Iwamoto Y; Yang K; Clifton G L; Hayes R L
CS Department of Neurosurgery, University of Texas Houston Health Science Center, Houston 77030, USA.
NC PO1 NS31998 (NINDS)
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SO NEUROREPORT, (1996 Jan 31) 7 (2) 609-12.

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TI Responses of young and aged rat CNS to partial cholinergic immunolesions and NGF treatment
AU Wortwein G; Yu J; ToliverKinsky T; PerezPolo J R (Reprint)
CS UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555 (Reprint); UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555; RIGSHOSP, LAB NEUROPSYCHIAT, DK-2100 COPENHAGEN, DENMARK
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SO JOURNAL OF NEUROSCIENCE RESEARCH, (1 MAY 1998) Vol. 52, No. 3, pp. 322-333.

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GA The Genuine Article (R) Number: 164UH
TI Nerve growth factor expressed in the medial septum following in vivo gene delivery using a recombinant adeno-associated viral vector protects cholinergic neurons from fimbria-fornix lesion-induced degeneration
AU Mandel R J (Reprint); Gage F H; Clevenger D G; Spratt S K; Snyder R O; Leff S E
CS LUND UNIV, WALLENBERG NEUROSCI CTR, NEUROBIOL SECT, SOLVEGATAN 17, S-22362 LUND, SWEDEN (Reprint); CELL GENESYS INC, DEPT PRECLIN BIOL, FOSTER CITY, CA 94404; SALK INST BIOL STUDIES, GENET LAB, LA JOLLA, CA 92037
CYA SWEDEN; USA
SO EXPERIMENTAL NEUROLOGY, (JAN 1999) Vol. 155, No. 1, pp. 59-64.

Shin-Lin Chen

Journal of Neuroscience Research 52:322–333 (1998)

Responses of Young and Aged Rat CNS to Partial Cholinergic Immunolesions and NGF Treatment

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The cholinergic neurons of the basal forebrain (CNBF) are the major source of cholinergic innervation of the cortex and hippocampus. In Alzheimer's disease and aged brain, there are severe losses of cholinergic neurons in the nucleus basalis of Meynert, leading to a reduction of cortical cholinergic activity which correlates with the severity of cognitive deficits. While there is evidence that aged central nervous system (CNS) displays impaired stress response signaling, pharmacologic treatments with neurotrophic factors appear to ameliorate these age-associated cholinergic deficits. To mimic these cholinergic deficits in experimental animals and study the acute effects of nerve growth factor (NGF), we induced a partial lesion of CBFNs by the intracerebroventricular (icv) injection of the cholinergic immunotoxin 192IgG-saporin, in groups of 3- and 30-month-old rats. The lesion was followed 14 days later by icv administration of NGF, known to restore partial immunolesion-induced cholinergic deficits in rat CNS, and all rats were killed 2 days after the NGF treatement. Here we report the effects of partial immunolesions on the levels of choline acetyltransferase (ChAT) activity and NGF receptor mRNA levels in the basal forebrain of 3- and 30-month-old rats. Because of their presence in the promoters of the NGF, NGF receptors, and ChAT genes, we also measured DNA-binding activity of the transcription factors NFB and AP-1 in the cortex and hippocampus. We discuss these findings in the context of endogenous NGF-mediated signal transduction mechanisms and conclude that we have evidence for age-associated decreases in endogenous NGF responses to partial deafferentation of the basal forebrain cholinergic projections. J. Neurosci. Res. 52:322–333, 1998.

Key words: 192 IgG-saporin; nerve growth factor; ageing; choline acetyltransferase; p75^{NGFR}; trkA; NFB; AP-1

INTRODUCTION

The cholinergic neurons of the basal forebrain (CBFNs) are the major source of cholinergic innervation of the cortex, hippocampus and olfactory bulb (Mesulam et al., 1983). There is ample evidence for a dependence of these neurons on nerve growth factor (NGF) during development and recovery from trauma (Auburger et al., 1987; Hefti, 1986; Gage et al., 1990; Hefti et al., 1993; Lindsay et al., 1994). While NGF is synthesized in the olfactory bulb, cortex, and hippocampus, which are target areas of CBFNs, NGF receptors are synthesized in the soma of CBFNs, anterogradely transported to the nerve terminals in the mentioned target areas where the receptors bind, internalize, and retrogradely transport the bound NGF to the cell body (Korsching et al., 1985; Schwab et al., 1979; Seiler and Schwab, 1984), where it is thought to exert its neurotrophic action. There are age-related declines in the function of CBFNs as measured by choline acetyl transferase (ChAT) activity and acetylcholine synthesis and release, and the CBFNs cell numbers (for review see Rylett and Williams, 1994). In Alzheimer's disease (AD) CBFNs undergo similar but more profound changes (i.e., Wilcock et al., 1982). There is a correlation between behavioral deficits and age-related declines in hippocampal ChAT activity and the loss of CBFNs (i.e., Fischer et al., 1987). In part, these deficits can be reversed by the administration of exogenous NGF at pharmacological concentrations (i.e., Fischer et al., 1987; Markowska et al., 1994). Although the ultimate causes of the age-associated loss of cholinergic function are not known, it has been widely speculated that failure of trophic function by NGF or other neurotrophins is

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responsible for or contributes to the CBFN functional losses that characterize age-associated cognitive deficits.

In agreement with this hypothesis, there is reduced NGF binding capacity in the aged rat's basal forebrain, hippocampus, and cortex (Angelucci et al., 1988; Albrecht et al., 1991). More recently, reduced transport of [^{125}I]NGF and reduced levels of trkA, but not p75^{NGFR} mRNA, in the basal forebrain of aged rats have been reported (Cooper et al., 1994). As to NGF protein levels, there are reports of decreases, increases, or no changes in the basal forebrain and its target areas in aged rats (Crutcher and Weintgartner, 1991; Albrecht et al., 1991; Hellweg et al., 1990; Larkfors et al., 1987) and patients with Alzheimer's disease (Allen et al., 1991; Crutcher et al., 1993). Taken together, these data suggest that there may be impairments of NGF-mediated signaling pathways in the basal forebrain cholinergic system during normal as well as pathological aging processes not reflected in measurements of basal levels of the neurotrophin and their receptors.

As discussed above, NGF is taken up by terminals of CBFNs in the hippocampus, cortex, and olfactory bulb and retrogradely transported to the cell bodies in the basal forebrain (Schwab et al., 1979; Seiler and Schwab, 1984; Altar and Bakhit, 1992; Ferguson et al., 1991; Lapchak et al., 1993; Korsching et al., 1985; Cooper et al., 1994). In primary septal cultures, NGF stimulates ChAT activity and promotes the survival of cholinergic neurons (Takei et al., 1988; Downen et al., 1993). In vivo, the intracerebroventricular infusion of pharmacological doses of NGF rescues CBFNs from degeneration after lesions of the fimbria fornix (i.e., Hefti, 1986) and restores reduced cholinergic function in aged rats (i.e., Fischer et al., 1987; Markowska et al., 1994). It has also been demonstrated that exogenous NGF up-regulates its own receptor (Higgins et al., 1989; Fusco et al., 1991; Koliatsos et al., 1994; Venero et al., 1994). In situ hybridization studies have shown that chronic NGF administration not only increases NGF receptor mRNA concentration within those CBFNs that normally express NGF receptor protein, but also induces the expression of NGF receptor message within basal forebrain neurons that normally do not contain detectable amounts of NGF receptor mRNA (Higgins et al., 1989). However, the signaling pathways by which NGF produces these effects have not been elucidated.

Recently it was reported that NGF selectively activates the transcription factor NFκB via the low-affinity neurotrophin receptor p75^{NTR} (Carter et al., 1996). NFκB is constitutively expressed in brain, where it has been localized to neurons and synaptic terminals (Kaltschmidt et al., 1993, 1994). When activated by external stimuli, NFκB translocates to the nucleus where it binds DNA and regulates gene transcription (Baeuerle and Henkel, 1994). The choline acetyltransferase gene sequence from *mus musculus* has potential NFκB, and AP-1 binding sites in its promoter region (Pu et al., 1993) and the NGF gene itself has a functional AP-1 binding site for the Fos/Jun heterodimer in the first intron (D'Mello and Heinrich, 1991). These findings make NFκB and AP-1 interesting targets in studies of the effects of NGF on the CBFN projection system.

A number of lesion paradigms have been used to examine NGF effects on the basal forebrain cholinergic system, such as fimbria-fornix transections and the injection of various toxins into the basal forebrain nuclei, their target areas, or the cerebral ventricles. One of the drawbacks of many of these lesion paradigms is their lack of specificity for the NGF-sensitive cholinergic neurons of the basal forebrain (for review see Smith, 1988). This has made it difficult to study the effects of neurotrophin responses to injury in this particularly relevant population of neurons. For this reason, we chose to use the immunotoxin 192IgG-saporin complex; 192IgG-saporin (192IgG-SAP) is made up of the 192IgG monoclonal antibody against the low-affinity NGF receptor, p75^{NTR}, which is coupled to the ribosome inactivating protein, saporin. 192IgG-SAP has been shown to selectively target p75^{NTR}-bearing CBFNs (Wiley et al., 1991; Heckers et al., 1994). Recently we developed an animal model that uses 192IgG-SAP to create a partial lesion of the CBFNs that reduces the number of p75^{NTR}-immunoreactive cells in the various nuclei of the basal forebrain by 40-60% and decreases ChAT activity in the cortex and hippocampus accordingly (Roβner et al., 1996). This partial 192IgG-SAP lesion model seems to be particularly well suited to study the responses of the remaining CBFNs to exogenous NGF treatment.

Here we report on the effects of intracerebroventricular (icv) NGF administration on the DNA binding activity of the transcription factors NFκB and AP-1 in cortex and hippocampus and on levels of mRNA for the NGF receptors p75^{NTR} and trkA in the basal forebrain of young and aged rats with a partial lesion of the CBFNs. We also measured ChAT activity in various areas of the brain as an index of the efficacy of the partial lesion in both young and aged rats.

MATERIALS AND METHODS

Animals and Surgical Procedures

Twenty-four 3-month-old and twenty-four 30-month-old male Fisher-344 Brown Norway hybrid rats (F344XBN; Harlan Sprague-Dawley Inc., Indianapolis, IN) were used in this study. Procedures, dosages, location of injections in young and aged rats, as well as time intervals (14 days) required for the full development of the immunolesions were based on published work (Roβner et al., 1995, 1996; Yu et al., 1995, 1996) or determined in

a preliminary experiment using the same 192IgG-SAP lot used in these experiments where efficacy of immunolesion was monitored using AChE histochemistry. Animals were anaesthetized with ketamine (60 mg/kg i.m. for young and 30 mg/kg i.m. for aged rats) and Nembutal (40 mg/kg i.p. for young and 30 mg/kg i.p. for aged rats), and placed in a Kopf stereotaxic apparatus. The skull was exposed and two small burr holes were made using a dental drill. A Hamilton syringe with a 26-gauge needle was used for injection. A 1.7 g/l stock solution of 192IgG-SAP (Chemicon Inc., Temecula, CA) was stored in frozen aliquots until just before use. Aliquots were then thawed and diluted to 0.65 g/l in sterile phosphate-buffered saline (PBS). Sixteen young and 16 aged animals received icv injections of 2 l of the diluted solution (1 l per side) at a rate of 1 l/min at the following coordinates: a/p: -0.8 mm (3 month old) or -1.0 mm (30 month old), m/l: +/-1.2 mm (3 month old) or +/-1.5 mm (30 month old), d/v: -4.0 mm (both groups), with the incisor bar set at -2.5 mm. Eight control animals from each age group received injections of 2 l sterile PBS at the same stereotaxic coordinates. After each injection the needle was left in place for 5 min to allow diffusion. The needle was then slowly retracted, the wound was bone-waxed and sutured, and the animal allowed to recover on a heating pad. No special postoperative care was required after 192IgG-saporin lesions in both young and aged rats.

Fourteen days after the 192IgG-saporin injections, eight 192IgG-saporin-injected animals from each age group received an icv injection of 5.4 g May 26, 1997-NGF in 6 l of PBS (3 l/side) at a rate of 1 l/min at the same coordinates and following the same general procedures as for the previous surgery. At the same time, all remaining animals received icv injections of 6 l of PBS (3 l/side) in the same manner as the May 26, 1997 NGF-treated animals. Thus, the subjects of this experiment fall into six different groups, each containing eight animals. Group PBS-PBS^{y} consists of 3-month-old animals that received icv PBS injections followed 14 days later by another icv PBS injection. Group SAP-PBS^{y} consists of 3-month-old animals that received icv 192IgG-SAP injections followed 14 days later by icv PBS injections. Group SAP-NGF^{y} consists of 3-month-old animals that received icv 192IgG-SAP injections followed 14 days later by icv NGF injections. Groups PBS-PBS^{a}, SAP-PBS^{a}, and SAP-NGF^{a} consist of 30-month-old animals that received injections corresponding to those received by groups PBS-PBS^{y}, SAP-PBS^{y}, and SAP-NGF^{y}, respectively.

Two days after the second surgery, all animals were killed by decapitation under deep halothane anesthesia and the brains were rapidly removed onto an ice-cold plate. Brain regions including the basal forebrain area, olfactory bulbs, bilateral hippocampus, and bilateral parieto-occipital cortex were dissected on ice. The tissues were immediately frozen on dry ice and stored at -80°C until processing. The basal forebrain area of four animals of each experimental group, as well as the olfactory bulb, one side of the hippocampus and one side of the cortex of all eight animals of each experimental group were used for ChAT and NGF determinations. The other side of the hippocampus and cortex of four animals from each experimental group were used for NFκB and AP-1 measurements by electrophoretic mobility shift assays (EMSA). The basal forebrain area of the remaining four animals of each experimental group was used for measuring levels of p75^{NTR} and trkA mRNA by ribonuclease protection assay (RPA).

Choline Acetyl Transferase Activity Determination

A 2-l aliquot of homogenate for the NGF ELISA was added to 58 l of buffer containing 10 mM EDTA and 1% Triton X-100. ChAT activity was determined by the method of Fonnum (1975) as described earlier (Yu et al., 1996). This method measures the conversion of [^{14}C]-acetyl-co-enzymeA to [^{14}C]acetylcholine. Briefly, tissue homogenates were incubated with the reaction substrate containing 600 mM NaCl, 0.2 mM eserine sulfate, 40 mM disodium EDTA, 100 mM phosphate buffer, 18 mM choline chloride, 0.39 mM acetyl-CoA, and 80 M [^{14}C]acetyl-CoA (0.3 mCi/mmol). Tetraphenylboron in acetonitrile was used to extract the synthesized [^{14}C]acetylcholine which was measured using a scintillation counter (Beckman Instrument Inc., Irvine, CA). The results are given as cpm/mg tissue.

NGF Immunoassay

NGF protein levels were measured using a two-site ELISA as described earlier (Yu et al., 1996; Roβner et al., 1996). The assay utilizes two antibodies, a monoclonal antibody raised against mouse NGF and the same antibody conjugated with β-galactosidase (Boehringer-Mannheim Corporation, Mannheim, Germany). The tissue was sonicated in 3l/mg wet weight of extraction buffer (pH 7.0) containing 100 mM Tris-HCl, 400 mM NaCl, 1% (w/v) bovine serum albumin, 0.05% (w/v) NaN_3, 4 nM EDTA, and the proteinase inhibitors aprotinin and phenylmethylsulfonylfluoride (PMSF). The amount of NGF in each sample was calculated from a standard curve run on the same plate using purified mouse NGF. All six experimental groups were measured on the same plate for a given brain region in order to account for the interplate variability of the assay. The results are given as pg/100 mg tissue/wet weight.

Preparation of RNA and Ribonuclease Protection Assays for p75^{NTR} and trkA mRNAs

Total tissue RNAs were extracted using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) and

quantitated spectophotometrically. The RNA probes specific for rat $p75^{NTR}$ and trkA were generated as described previously (Yu et al., 1995). Briefly, the rat $p75^{NTR}$ specific probe was generated by transcribing the linearized plasmid pAN (Hutton et al., 1992) using T7 RNA polymerase, resulting in a 327-nucleotide-long probe. The specific hybridization product was 261 nucleotides long. The rat trkA specific probe was generated by restriction digestion of pDM115.203 (Hutton et al., 1992) and transcription of the linearized plasmid using T7 RNA polymerase. This gave rise to a 329-nt probe and a specific hybridization product of 203 nt. Internal control probe (28S) was prepared according to the manufacturer's instructions (Ambion Inc., Austin, TX). Hybridization of RNA probes with total RNA (30 g) from the basal forebrain area and ribonuclease digestion was carried out using the Ambion ribonuclease protection assay RPA II kit (Ambion Inc.). For quantification, the gels were dried and scanned with a phosphorimager. Phosphorimager units for the $p75^{NTR}$ and trkA mRNA specific bands were corrected for values obtained for 28S mRNA in that sample, to correct for potential nonspecific changes in mRNA levels that might have resulted from the experimental procedures. The value of young control animals was set at 100% for each gel and all other values are expressed as percentage of the corresponding control value.

Nuclear Extracts

Tissues were harvested as described by Toliver-Kinsky et al., (1997). Tissues were homogenized on ice in 3.5 l/mg tissue wet weight with a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT, 0.5 mM PMSF, and 2 g/ml each of antipain, chymostatin, pepstatin, and leupeptin. The lysate was centrifuged at 8,000 rpm for one minute at 4°C, and the nuclear pellet reconstituted in 1.5 l/mg wet weight of buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 2 g/ml each of antipain, chymostatin, pepstatin, and leupeptin, followed by vigorous vortexing for 20 min at 4°C. The nuclear lysate was centrifuged at 14,000 rpm for 5 min, and nuclear extracts aliquoted, frozen, and stored at -80°C. Nuclear proteins were quantitated using the Pierce® bicinchoninic assay (Pierce, Inc., Rockford, IL).

Electrophoretic Migration Shift Assay (EMSA)

Oligonucleotides containing the NFκB and AP-1 consensus sequence (SC-2505 and SC-2501, Santa Cruz Biotechnology, Santa Crz, CA) were end-labeled with ^{32}P-ATP (150 Curie at 6,000 Curie/mmole) using 8 units of T4 polynucleotide kinase and purified. Equal amounts of nuclear proteins (20 g) were incubated for 20 min at room temperature with 250 pg of labeled NFκB or AP-1 consensus sequence and 2 g poly (dI-dC)-poly (dI-dC) as a nonspecific competitor. For competitive controls, unlabeled consensus sequence was added to samples in 100-fold molar excess. The reaction mixtures were electrophoresed in a 5% acrylamide gel at 4°C, 120 volts, for 3 hours. Gels were dried and exposed to X-ray film, which was scanned and quantitated by a densitometer. In separate experiments reported elsewhere (Tong and Perez-Polo, 1995; Tong and Perez-Polo, 1996; Tong et al, 1997), the use of antibodies to AP-1 and NFκB subunits in "supershift" experiments and of a mutant form of the AP-1 DNA consensus sequence confirmed the identity of the AP-1 and NFκB 32P-labeled oligonucleotide bands displayed in Figures 5 and 6.

Statistical Analysis

Two-way analysis of variance (ANOVA) was used to determine statistically significant treatment and age effects at $P < 0.05$, followed by t-tests where appropriate. Also where appropriate a Kruskal-Wallis analysis of variance (one-way) was carried out instead, followed by Mann-Whitney U-tests.

RESULTS

ChAT Levels

Based on previous experience with the partial immunolesion paradigm (Roβner et al., 1996), bilateral icv injections of 0.65 g 192IgG-saporin, or PBS vehicle were followed 14 days later by 5.4 μg NGF icv or PBS vehicle were carried out on 3- and 30-month-old rats. Because of our specific interest in deafferentation vs. more general injury effects, all comparisons were made to sham-treated animals. All animals were killed 2 days after the NGF or PBS injections. As reported elsewhere (Roβner et al., 1996), 192IgG-saporin injections significantly reduced ChAT activity levels in the cortex, hippocampus ($P < 0.001$), and olfactory bulb ($P < 0.05$) but not the basal forebrain of 3-month-old rats when compared to sham-treated counterparts (Fig. 1). Interestingly, the icv bolus NGF treatment of 3-month-old lesioned or sham-lesioned rats had no discernible effect on ChAT activity after 2 days. When 30-month-old rats were treated similarly, there were significant lesion-induced decreases in ChAT activity in the aged cortex and hippocampus ($P < 0.05$) but not the basal forebrain and olfactory bulb when compared to the sham-treated 30-month-old rats and again NGF had no significant effect on ChAT levels 2 days after the lesion except for the cortex ($P < 0.05$). There were significant decreases in ChAT activity in the sham-treated 30-month-old rat cortex, basal forebrain ($P < 0.01$) and olfactory bulb ($P < 0.05$)

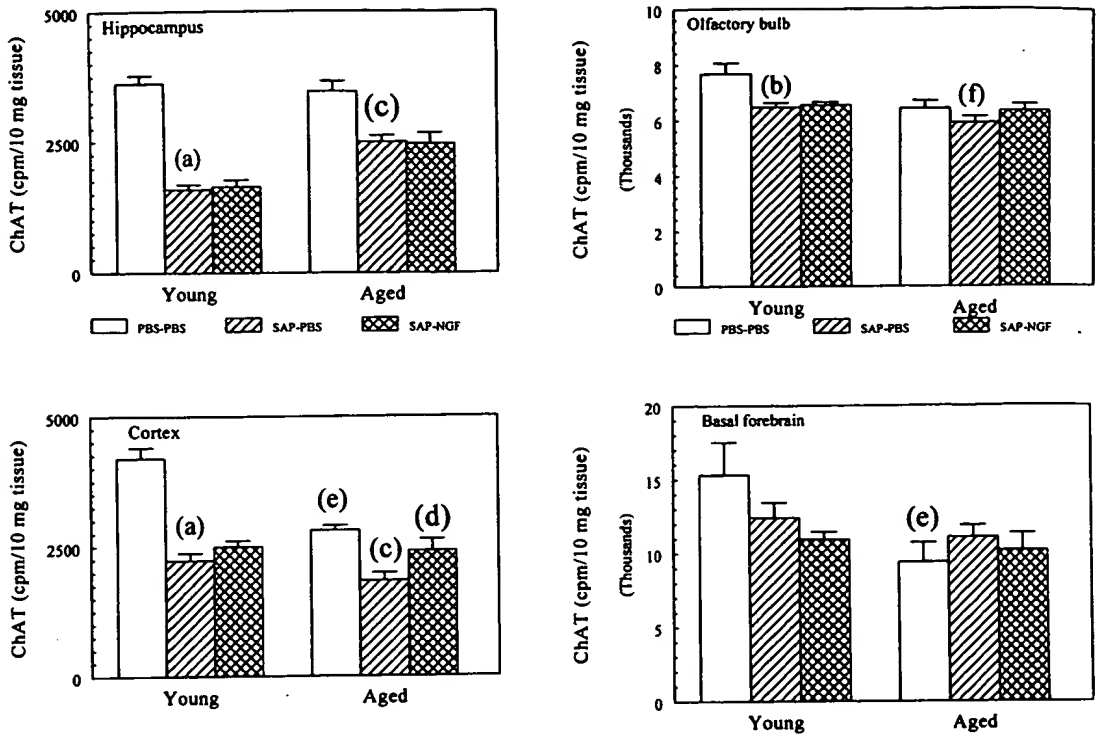

Fig. 1. Effect of 0.65 g 192IgG-Saporin (SAP) bilateral injection on ChAT levels measured 14 days after immunolesionsand a further 2 days in the presence or absence of NGF. (a) Difference between immunolesioned and sham-treated $P < 0.001$; (b) as in (a) except $P < 0.05$; (c) as in (a) for 30-month-old rats and $P < 0.05$; (d) difference between 30-month-old immunolesioned cortex compared to NGF-treated immunolesined rats $P < 0.05$; (e) difference between sham-treated 30-month-old and 3-month-old sham-treated $P < 0.01$; (f) effect of immunolesion on 30-month-old rats $P < 0.05$.

compared to 3-month-old sham-treated rats, but there were no significant differences between ChAT levels in immunolesioned 3- vs. 30-month-old rat cortex, basal forebrain or olfactory bulb.

NGF Protein Levels

NGF protein levels were then determined in brain regions of 3- and 30-month-old rats treated as described. The results of the NGF immunoassays are shown in Figure 2. As expected 16 days after partial immunolesions, brain tissue NGF protein levels were returned to normal (Roβner et al., 1996). There were also no age-associated changes in NGF protein levels, and immunolesioned rats treated with NGF showed significant increases in NGF protein levels.

In order to determine the fate of the exogenous 2 days after its injection, we measured NGF protein levels in the cerebrospinal fluid (CSF) of all rats (Fig. 3). Two-way ANOVA of CSF NGF levels showed that samples from immunolesioned 3-month-old rats were higher than sham-treated rats ($P < 0.05$) but CSF NGF levels in immunolesioned 30-month-old rats did not differ from NGF levels in their sham-treated counterparts. There were no significant differences in the concentrations of NGF in the CSF of 3- and 30-month-old sham-treated rats or between NGF-treated immunolesioned and immunolesioned alone 3-month-old rats.

P75^{NGFR} and trkA mRNA Levels

Given that NGF action is dependent on NGF receptor expression and that induction of NGF receptor mRNAs by NGF may be an element of stress response signal activation, we measured p75^{NTR} and *trk*A mRNA levels in the basal forebrain, where it is synthesized, of all rats used in our immunolesion experiments (Fig. 4). Kruskal-Wallis one-way analysis of variance revealed a significant effect of 192 IgG-saporin treatment on p75^{NTR} mRNA levels in the basal forebrain area of 3-month-old animals but not of 30-month-old animals when compared to sham-treated counterparts. Post-hoc Mann-Whitney U-tests showed significant differences between 3-month-old sham-treated and immunolesioned rats ($P < 0.05$) and between 3-month-old immunolesioned and NGF-treated immunolesioned rats ($P < 0.05$). Interestingly, NGF treatment of 3-month-old rats induced increases in p75 mRNA to levels in excess of those present in

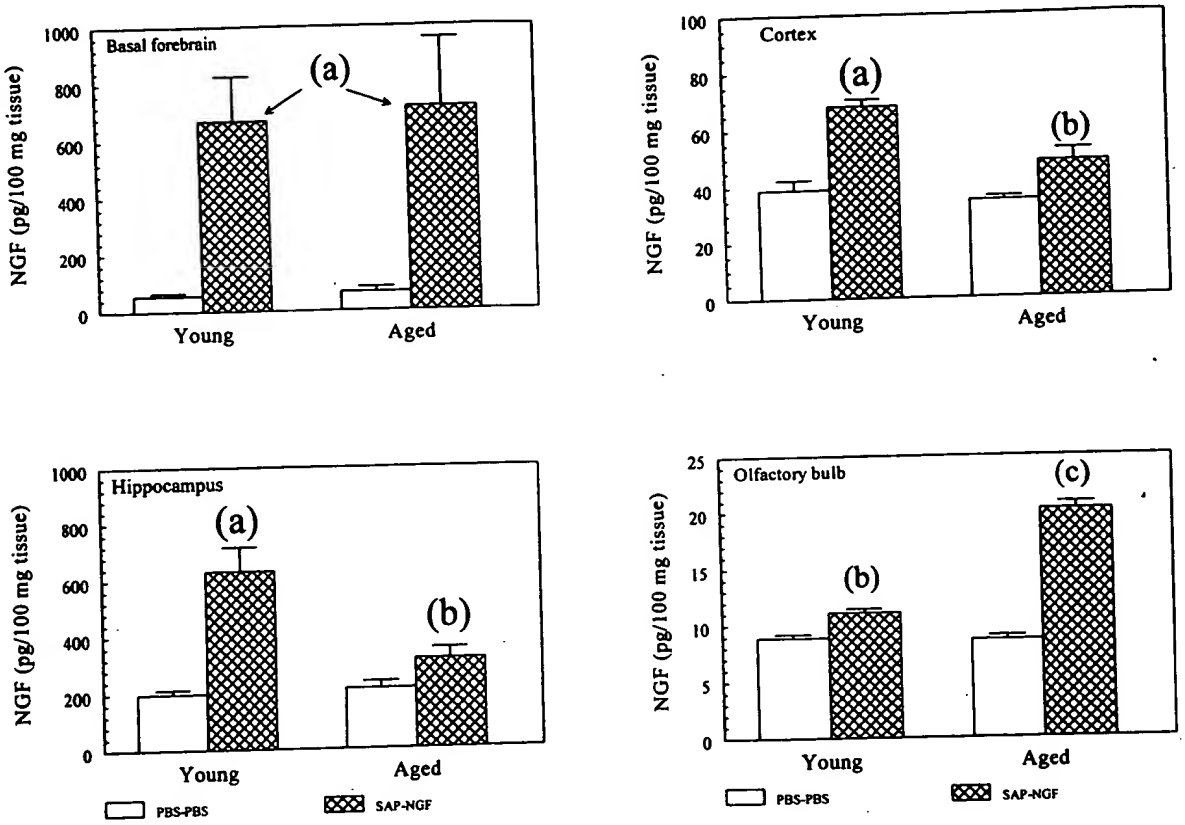

Fig. 2. Effect of 0.65 g 192IgG-Saporin (SAP) bilateral injection on NGF protein levels measured 14 days after immunolesions and a further 2 days in the presence or absence of NGF. (a) Difference between 3 month-old NGF-treated immunolesioned rats and sham-treated or immunolesioned alone $P < 0.01$; (b) as (a) except for 30 month-old rats $P < 0.05$; (c) as (a) except for 30 month-old rats $P < 0.02$.

sham-treated rats. There were no significant 192 IgG-saporin or 2-day NGF treatment effects on $p75^{NTR}$ or TrkA mRNA levels in the basal forebrain region of the 30-month-old animals and there were also no specific responses in $p75^{NTR}$ or TrkA mRNA levels in the basal forebrain of 30-month-old rats to partial immunolesions and a 2-day NGF treatment. Analysis of trkA mRNA levels showed a very similar pattern for both the 3- and 30-month-old rats.

NFB and AP-1 Binding Activity

Because of the presence of NFκB and AP-1 binding sites in the promoters for the NGF, $p75^{NTR}$, and ChAT genes, we assessed NFκB and AP-1 DNA binding activities. Electrophoretic migration shift-assays (EMSAs) of brain-derived nuclear extracts for NFκB DNA binding activity showed two specific bands consistent with previous reports (Toliver-Kinsky et al., 1997). Control experiments relying on the use of an excess of unlabeled probe or the use of mutant oligonucleotide sequences as probes (Tong and Perez-Polo, 1995) confirmed the specificity of the band shifts. It has been suggested

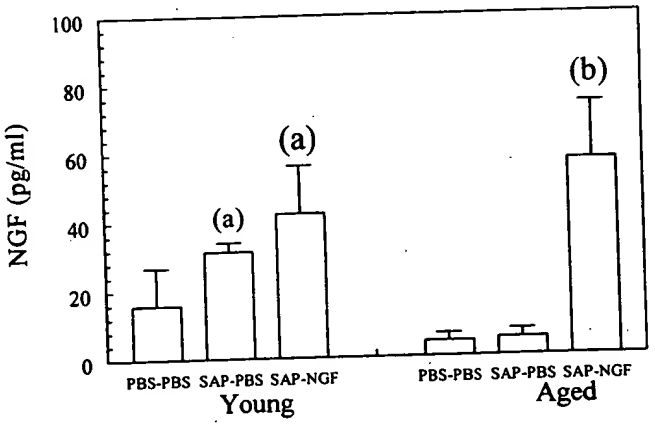

Fig. 3. Concentration of NGF in cerebral spinal fluid (CSF) after partial immunolesions (SAP) and NGF treatment. (a) NGF concentrations in 3-month-old immunolesioned rats were higher when compared to sham-treated $P < 0.02$; (b) NGF-treated immunolesioned rats $P < 0.001$ were higher than sham-treated, and NGF concentrations in 30-month-old NGF-treated immunolesioned rats were higher than in 30 month-old sham-treated $P < 0.001$. Thirty-month-old sham-treated rats had lower CSF NGF concentrations $P < 0.05$ compared to 3 month-olds.

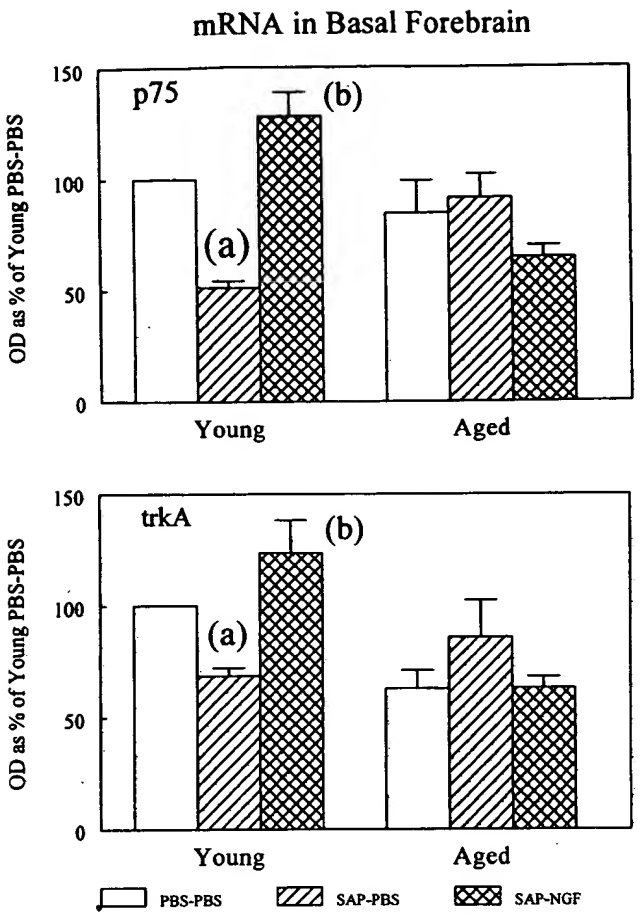

Fig. 4. Effect of 0.65 g 192IgG-Saporin (SAP) bilateral injection on NGF receptor mRNA levels measured after partial immunolesions in the presence and absence of NGF where (a) = $P < 0.05$ and (b) = $P < 0.05$.

that the higher molecular weight band corresponds to a p65-p50 complex bound to the labeled oligonucleotide and the lower molecular weight band corresponds to a p50-p50 complex. Two-way ANOVA revealed no significant age or immunolesion effects on NFκB binding activity in the cortex (data not shown). In the hippocampus, consistent with previous results (Toliver-Kinsky et al., 1997), aged rats consistently displayed increased NFκB DNA-binding activity ($P < 0.001$) associated with the higher molecular weight band (Figs. 5 and 6). Furthermore, while NFκB DNA-binding activity was minimally higher in the hippocampus of immunolesioned rats compared to sham-treated controls, NGF treatment augmented the immunolesion effect ($P < 0.01$), most dramatically in the aged immunolesioned hippocampus of NGF-treated rats ($P < 0.001$). A similar two-way analysis of variance of AP-1 DNA-binding activity in the cortex and hippocampus by EMSA showed reciprocal age-associated changes in AP-1 DNA-binding activity in cortex and hippocampus ($P < 0.001$ for cortex; $P < 0.01$ for hippocampus; Figs. 7 and 8). There were age-associated increases in AP-1 DNA-binding activity in cortices and decreases in hippocampus under all conditions. Immunolesions and NGF treatment had no effect on 3-month-old cortices but did decrease AP-1 binding ability in 30-month-old rats, an effect fully reversed by NGF treatment. In the hippocampus NGF treatment significantly decreased levels of AP-1 DNA-binding activity in the 30-month-old immunoleioned rats ($P < 0.01$).

DISCUSSION

As previously reported (Roβner et al., 1996), the icv infusion of 1.3g 192IgG-SAP in 3-month-old Sprague-Dawley rats causes a substantial (40-60%) but less than maximal decrease in cortical and hippocampal ChAT activity similar to the decreases in ChAT activity observed here using 3-month-old male F344XBN hybrid rats. For the 30-month-old F344XBN rats, administration of the same amount of immunotoxin resulted in a decrease in ChAT activity to levels that were very similar to those obtained for their 3-month-old counterparts (Fig. 1). Given that the sham-treated 30-month-old rats exhibited a very significant decrease in ChAT, consistent with other reported measurements of basal levels of ChAT in untreated aged rats, the net differential decrease in ChAT for the immunolesioned 30-month-old rats when compared to their sham-treated counterparts was not as large as that observed in the immunolesioned 3-month-old rats. Whether decreases in ChAT activity in aged rats is due to decreases in the number of cholinergic neurons, decreases in cholinergic expression per cholinergic neuron, or a combination of both factors cannot be determined from these experiments. However, given that NFB activity is increased in aged F344XBN rat CNS and that NFB can act as a repressor of ChAT activity (Toliver-Kinsky et al., 1997), the two latter explanations are more likely.

The observation that the NGF levels in the 30-month-old rats did not significantly differ from their 3-month-old counterparts, agrees with observations showing that NGF protein levels are not altered in aged rats in other strains (Scott et al., 1994; Crutcher and Weingartner, 1991). One study examined potential strain differences in the age-related changes of hippocampal and cortical NGF levels and found more pronounced decreases in Fischer-344 and Brown Norwegian rats as compared to Sprague-Dawleys (Larkfors et al., 1987b). Others have correlated NGF levels with the cognitive status of aged rats and found no change or even slight increases in NGF levels in the various brain areas of moderately to severely impaired aged rats (Hellweg et al., 1990). To our knowledge, these are the first published

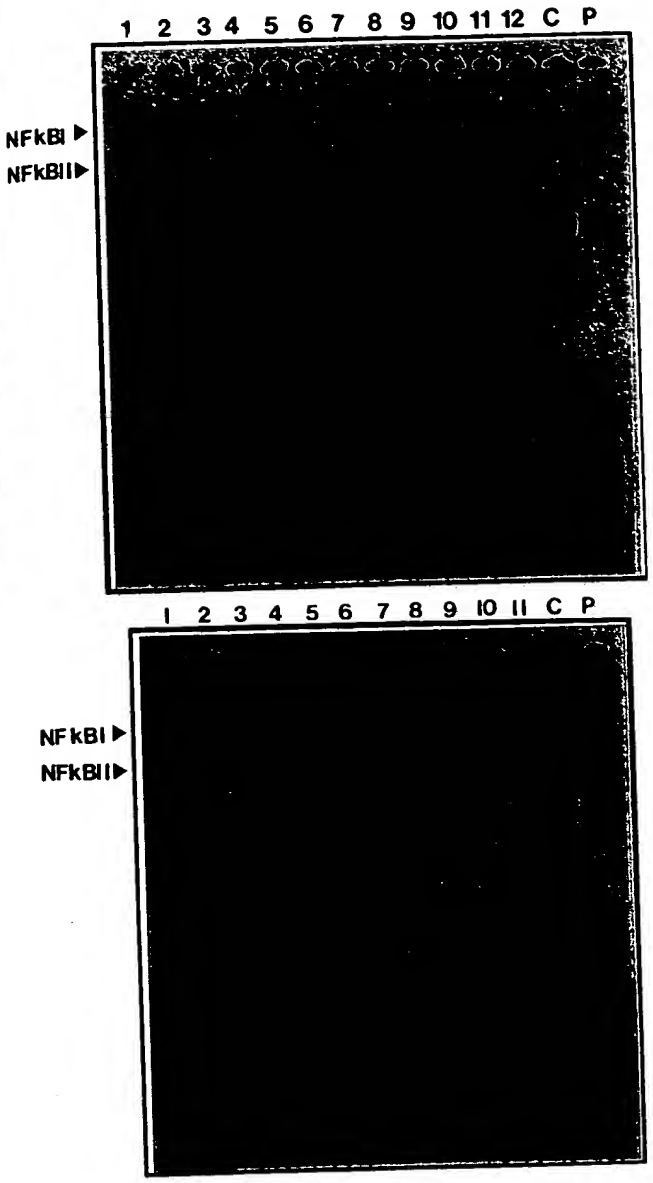

Fig. 5. Effect of 0.65 g 192IgG-Saporin (SAP) bilateral injection on NFB DNA-binding activity in hippocampal nuclear extracts measured 16 days after immunolesions and 2 days after NGF treatment as measured by EMSA. **Upper** panel shows EMSAs from 3-month-old rats: **lanes 1-4**, PBS-PBS-treated rats; **lanes 5-8**, SAP-PBS-treated rats; **lanes 9-12**, SAP-NGF-treated rats; C, competitive control; P, probe alone. **Lower** panel shows EMSAs from 30-month-old rats: **lanes 1-3**, PBS-PBS-treated rats; **lanes 4-7**, SAP-PBS-treated rats; **lanes 8-11**, SAP-NGF-treated rats; C, competitive control; P, probe alone.

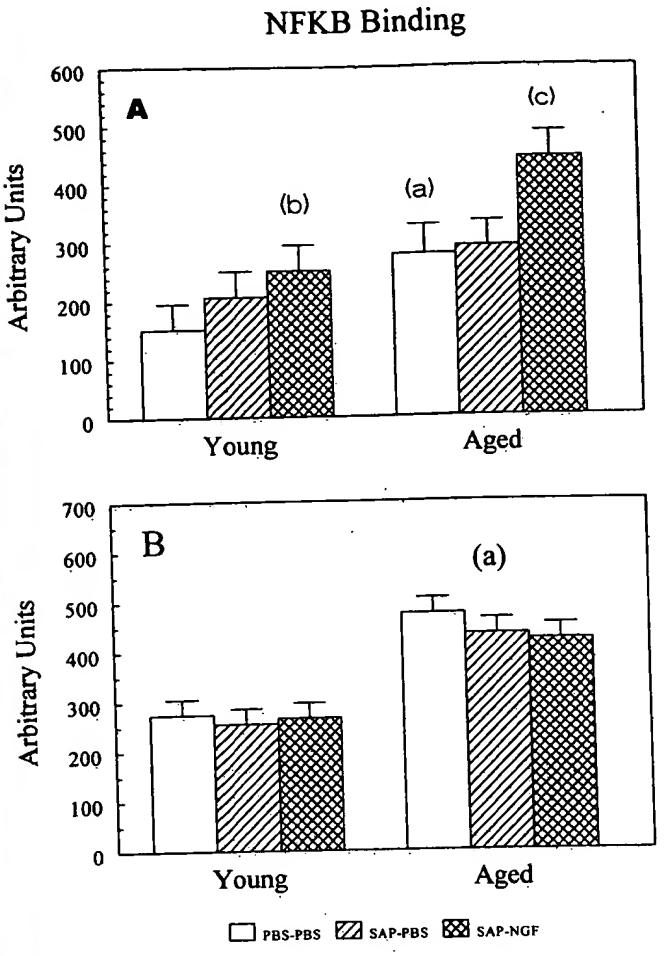

Fig. 6. Quantitation of autoradiographs of EMSAs depicted in Figure 5. **A:** DNA-binding activity significantly higher in the 30-month-old sham-treated rats compared to their 3-month-old counterparts, $P < 0.001$ (b). NGF effect on 30 month-old immunolesioned rats compared to sham-treated counterparts $P < 0.001$ (a). **B:** NGF effect on 3-month-old immunolesioned rats compared to sham-treated $P < 0.01$ (a).

measurements of NGF protein levels in the brains of young or aged F344XBN hybrids.

Our finding that a single injection of NGF had no effect, after only 2 days, on ChAT levels in the basal forebrain of immunolesioned rats, young or old, is consistent with our previous report of an NGF stimulation of ChAT activity in young Sprague-Dawley rats after 30 days (Roβner at al., 1996) and other studies showing NGF stimulation of ChAT activity in lesioned CNS (Vantini et al., 1990; Williams et al., 1993; Chen and Gage, 1995). It is likely that induction times of more than 2 days may be necessary to observe NGF effects on ChAT activity under the very selective and mild injury paradigm implicit in partial immunolesions. This interpretation also suggests that while the partial immunolesion paradigm is likely to not be useful to the analysis of injury or disease responses, it may be a good model for aging processes in the absence of disease.

Consistent with previous results, the partial immunolesion significantly reduced the amount of $p75^{NTR}$ and trkA mRNA levels. That the decrease in trkA mRNA was

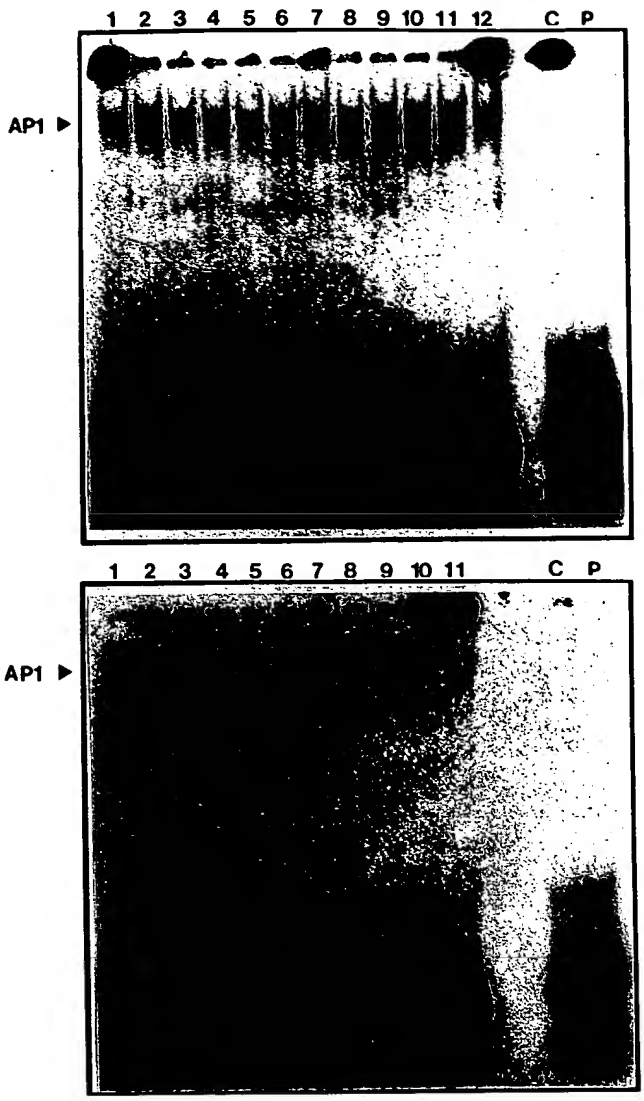

Fig. 7. Effect of 0.65 g 192IgG-Saporin (SAP) bilateral injection on AP-1 DNA-binding activity in hippocampal nuclear extracts measured 14 days after immunolesions and 2 further days in the presence or absence of NGF as measured by EMSA. **Upper** panel shows EMSAs from 3-month-old rats: **lanes 1-4**, PBS-PBS-treated rats; **lanes 5-8**, SAP-PBS-treated rats; **lanes 9-12**, SAP-NGF-treated rats; C, competitive control; P, probe alone. **Lower** panel shows EMSAs from 30-month-old rats: **lanes 1-3**, PBS-PBS-treated rats; **lanes 4-7**, SAP-PBS-treated rats; **lanes 8-11**, SAP-NGF-treated rats; C, competitive control; P, probe alone.

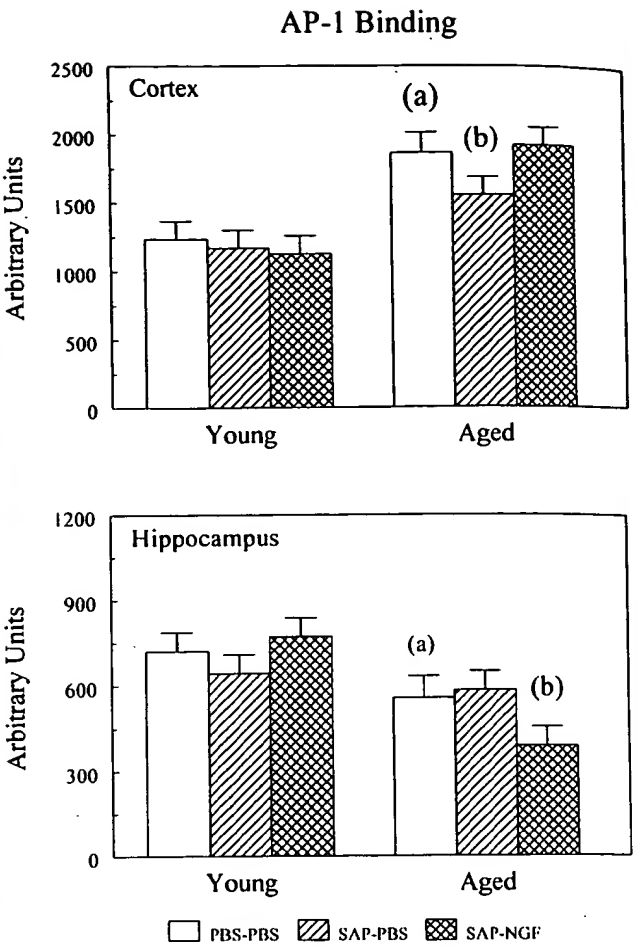

Fig. 8. Quantitation of autoradiographs of EMSAs depicted in Figure 7 (a). DNA-binding activity significantly higher in the 30-month-old sham-treated rats compared to their 3-month-old counterparts, $P < 0.001$ and immunolesion effect on 30-month-old rats compared to sham-treated counterparts $P < 0.01$. (b) NGF effect on 3-month-old immunolesioned rats compared to sham-treated $P < 0.01$.

not as pronounced as the $p75^{NTR}$ mRNA effects is likely due to the presence of three kinds of NGF-responsive neurons displaying $p75^{NTR}$, $p75^{NTR}$ and TrkA, and TrkA alone, where only the first two neuronal cell types are affected by 192IgG-saporin immunolesions. Interestingly, NGF treatment restored both $p75^{NTR}$ and TrkA mRNA levels in excess of levels observed for sham-treated rats in agreement with the reported NGF-dependent stimulation of NGF receptors in the adult rat CNS (Higgins et al., 1989; Fusco et al., 1991.

Messenger RNA levels for TrkA, but not for $p75^{NTR}$ were decreased in the basal forebrain area of 30-month-old as compared to 3-month-old F344XBN sham-treated rats. This is consistent with previous findings in Sprague-Dawleys (Cooper et al., 1994). The 192IgG-SAP-induced decrease in $p75^{NTR}$ and trkA mRNAs in the 3-month-old rats was reversed by icv NGF treatment within 2 days. In the 30-month-old animals, in contrast, neither the lesion nor the NGF treatment had significant effects on the mRNAs for either receptor. This can be explained on the basis of the observed reduced binding capacity of NGF in the basal forebrain and hippocampus of aged Sprague-Dawley rats (Angelucci et al., 1988) and aged cortex

(Albrecht et al., 1991). The absence of a response to the lesion or to the NGF treatment in receptor mRNA levels in the aged rats could be due to age-related impairments of the pathways regulating $p75^{NTR}$ and trkA expression at the transcriptional level.

Further support for this hypothesis comes from our observation of altered DNA-binding levels of the transcription factors NFκB and AP-1 in the aged rat hippocampus but not cortex. While NFκB DNA-binding activity was consistently higher in the hippocampus of aged rats of all experimental groups, no such increases were observed in the cortex consistent with previous observations. Not surprisingly whereas immunolesions had no effect on NFκB DNA-binding levels 16 days after the event, NGF administration significantly increased NFκB DNA-binding in both age groups in the hippocampus, but not the cortex by 2 days. It has to be emphasized that these measures of NFκB activity refer to a global effect and need to be further dissociated into effects on individual gene promoters. The NGF effect on NFκB was most pronounced in the aged rat hippocampi consistent with an exaggerated response of aged brain to NGF. These results would further suggest that the absence of NGF effects on ChAT activity after 2 days may reflect the short time at which animals were sacrificed after NGF treatment, as required for the more transient NFκB and AP-1 measurements, the more central aspects of this study, as opposed to the effects of NGF on ChAT, already well documented. Since it has been reported that NGF stimulates activation of NFκB in cultured Schwann cells via a $p75^{NTR}$ mediated process, it is not surprising to see that the effects on NFκB resemble the $p75^{NTR}$ effects (Carter et al., 1996). Taken together, these results suggest that NFκB participates in the mediation of some of the effects of NGF. The nature of the NGF effects mediated by NFκB or the specific genes so regulated remain to be elucidated. For example, NFκB stimulates the transcription of the amyloid-beta-protein-precursor (APP) in cells of neural origin while repressing ChAT expression (Grilli et al., 1996; Toliver-Kinsky et al., 1997).

The observation that NFκB DNA-binding activity is higher in the hippocampi of aged rats may reflect a requirement for survival of higher levels of constitutive NFκB DNA-binding activity in response to age-related increases in stress and apoptotic cell death (Taglialatela et al., 1996). This increase in NFκB activity may be anti-apoptotic given that NFκκB levels can regulate cellular commitment to apoptosis by PC12 cells (Taglialatela et al., 1997).

We observed no significant effects of immunolesions on AP-1 DNA-binding activity consistent with the transient nature of AP-1 changes and our killing of rats 16 days after immunolesions. AP-1 binding was somewhat increased in the cortex of aged rats, but decreased in the hippocampus, most prominently in the aged hippocampus where NGF treatment further depressed the AP-1 binding levels. NGF significantly increased AP-1 DNA-binding in the aged cortex at 2 days, again reflecting reported age-associated hypersensitivity in the CNS. This is an interesting finding since the augmentation of NGF synthesis provoked by phorbol-12-myristate 13-acetate treatment has been reported to be due to recruitment of AP-1 (Jehan et al., 1996). Thus, NGF-induced depression of AP-1 binding in the aged hippocampus, as observed in this study, could be due to a negative feedback mechanism. Our finding of mild decreases in AP-1 activity in the aged hippocampus in control animals is in agreement with other reports (Kaminska and Kaczmarek 1993; Toliver-Kinsky et al., 1997). Our observation of elevated AP-1 DNA-binding in the aged cortex raises several interesting issues. AP-1 is increased by many different stimuli in the brain, including events that are known to result in the generation of free radicals (Morgan and Curran, 1991; Sonnenberg et al., 1989; Kaminska et al., 1996; Moore et al., 1996; Williams and Jope, 1995). Since free radicals are implicated in many of the age-related or injury-induced changes in the brain, it is likely that the increased AP-1 activity observed in the cortex of our aged animals correlates with the increased level of free radicals there, while the increased NFκB activity present in the aged hippocampus may serve a protective function for this structure from age-associated stresses and free radical-mediated events that result in apoptosis. Again, it must be remembered that AP-1 acts as a summation point for the action of may different transcription factors and that minor DNA binding site sequence differences and site-site interactions in the regulation of different genes may account for vastly different effects on different genes by one transcription factor.

Sixteen days after a partial immunolesion, we observed no effects of the lesion as such on NFκB or AP-1 DNA-binding activity in the cortex or hippocampus of young as well as aged animals. This finding is in agreement with reports of the time course of NFκB DNA-binding activity after traumatic brain injury, where NFκB activity completely subsides 10 days after the injury (Yang et al., 1995).

In conclusion, we consider these findings consistent with the hypothesis that NGF-mediated stress or injury responses in the aged are impaired as reflected by altered NFκB and AP-1 signal transduction pathways with consequences on NFκB and AP-1 regulated genes, such as ChAT, NGF, and its receptors.

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